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Thermal Damage Assessment of Blood Vessels in a Hamster Skin Flap Model by Fluorescence Measurement of a Liposome-Dye System

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Background and Objectives: **The present study was undertaken to evaluate the feasibility of thermal damage assessment of blood vessels by using laser-induced release of liposome-encapsulated dye.**
Study Design/Materials and Methods: **Experiments were performed in a hamster skin flap model. Laser irradiation was achieved with a 300 μm fiber connected to a 805 nm diode laser (power=0.8W, spot diameter=1.3 mm and pulse exposure time lasting from 1 to 6 s) after potentiation using a specific indocyanine green (ICG) formulation (water and oil emulsion). Liposomes-encapsulated carboxyfluorescein were prepared by the sonication procedure. Carboxyfluorescein (5,6-CF) was loaded at high concentration (100 mM) in order to quench its fluorescence. The measurements were performed after i.v. injection of DSPC liposomes (1.5 ml) and lasted 40 min. Fluorescence emission was measured with an ultra high sensitivity intensified camera.**
Results: **Three different shapes of fluorescent spots were identified depending on target (blood vessel or skin) and energy deposition in tissue: (i) intravascular fluorescence, (ii) transient low fluorescence circular spot, and (iii) persistent high intense fluorescence spot. These images are correlated with histological data.**
Conclusion: **Real-time fluorescence imaging seems to be a good tool to estimate in a non-invasive manner the thermal damage induced by a diode laser combined with ICG potentiation. Lasers Surg. Med. 20:131–141, 1997.**

Key words: temperature-sensitive liposomes; 805nm diode laser; blood vessel; skin indocyanine green

INTRODUCTION

Methods for thermal damage assessment during and after laser photocoagulation are usually required for selective destruction of tissue. The "gold standard" technique is histology. But this technique requires a tissue section and the damage is quantified after an important delay. Since the tissue damage is defined as the denaturation or loss of function of biological molecules found in cells or extracellular fluid due to laser-induced heating, and since in most cases a non-invasive procedure is required, several techniques have been proposed. The first one consists of estimating the time-temperature history by

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measuring the surface tissue temperature using infrared radiometry [1]. Since the surface temperature is measured in real-time, the damage can be estimated quasi-instantaneously by numerical integration using the temperature response and empirical rate coefficients. Pulsed photothermal radiometry (PPTR) provides also a non-invasive and very fast (MHz) means to estimate the initial temperature distribution in tissue. For example, Milner et al. [2] have shown that PPTR gives useful port-wine stains diagnostic information. The second one is based on analysis of reflectance since photocoagulation usually produces an increase of reflectance as the tissue turns white. However, in most cases the technique is restricted to superficial tissue damage assessment. The use of measured reflectance increase due to the laser irradiation is characterized by a latency period after the initiation of the irradiation, during which no reflection has occurred [3]. Consequently, the reflection rise does not reliably predict the coagulation effects from the initial phase of the reflection change. Substantial variations are observed and it seems that reflectance becomes a good parameter only as soon as tissue temperature is superior to 70°C [4].

We have already proposed a new technique based on release of a dye encapsulated in temperature-sensitive liposomes [5,6]. This technique consists in encapsulating at high concentration a fluorescent dye into the liposomes in order to quench its fluorescence. These liposomes are injected intravenously. During the heating of liposomes up to their phase-transition temperature, a leakage of the dye is observed. This leakage leads to a dequenching of the fluorescence which can be observed. This fluorescence emission depends on the amount of dye released which is a function of the temperature. Usually based on the liposome composition, it is possible to define different temperature ranges [7]. Using DSPC liposomes, we have demonstrated the feasibility of this new technique in vivo in liver, and the possibility to measure temperature in the range 42–65°C. Since this temperature range corresponds to the coagulation temperature range of most tissues, we were interested in evaluating this new technique for blood vessel thermal damage assessment.

Selective destruction of blood vessels is an important application of lasers particularly in ophthalmology and dermatology. The ability to close deep vessels would provide an important tool for the treatment of abnormal choroidal vessel and of angiodysplasias of the skin. Occlusion of large vessels would allow treatment of tumors as well. In dermatology, damaging blood vessels by heating hemoglobin with a yellow laser beam is relatively successful for the treatment of vascular lesions. Two factors limit this technique: (i) specificity for thermal damage is limited by the absorption of light by melanin, and (ii) an insufficient depth of light penetration prevents thick lesions from being successfully treated.

Recently a new approach has been proposed. This approach requires a laser which is not absorbed by the biological tissue but by a dye specifically located in the blood vessel. It has been suggested that ICG dye enhanced diode laser photocoagulation could achieve more selective treatment of blood vessels with less damage to adjacent tissular structures [8]. The principle is based on the fact that there are no absorption sites for 805nm radiation within the biological tissues. Since the emission and absorption peaks of the diode laser and ICG are similar, ICG dye-enhanced diode laser photocoagulation may allow selective ablation of the ICG-containing vessel. It should be emphasized that this technique is experimental at present: Reichel et al. [9] have reported one successful treatment of a patient with peripheral retinal telangectasia and vitreous hemorrhage. However, if this technique is interesting, it has one main constraint which depends on the short half-life of ICG. Consequently, the laser treatment was performed almost immediately after intravenous administration of ICG and was completed within 2 to 3 min after initiation of photocoagulation. An administration method that maintained a high steady state plasma concentration of ICG was proposed by Obana et al. to alleviate this problem [10]. But if the treatment lasts, the leakage of dye from the vessels reduces the blood vessel selectivity. Consequently Ho et al. have suggested that these two restrictions, (i) the very-short half-life of ICG in blood and (ii) the lack of selectivity in blood vessels, may be a relative contraindication for ICG dye-enhanced laser photocoagulation [11].

The problem is identical in dermatology. Bass has reported the treatment of several patients with angiodysplasias using systemic ICG injection or intralesional ICG injection and 805 diode laser irradiation [12]. The results were encouraging but the impossibility to assess in real-time the selectivity of blood vessel thermal damage was a major limitation due to the short half-life of ICG and the lack of selectivity after a few minutes. We have also demonstrated (data not

shown) that an effective conversion of light into heat is obtained only for less than 5 min after ICG injection. Incorporation of ICG in emulsion system may be an alternative way to increase the circulating half-life of ICG and to confine this dye in a particle displaying high absorption and fluorescence properties. Moreover, the incorporation of ICG in emulsion may confine ICG in the vascular compartment and limit the interaction of ICG with blood components. Emulsions formulations (water and oil: o/w) have been proposed to obtain long circulating systems for highly lipophilic drugs [13]. We have already demonstrated in a preliminary report that ICG could be incorporated in emulsion [14,15]. A threefold increase of ICG concentration in blood was measured in rats 30 min after injection of the emulsion compared to the free dye injection.

The present study was undertaken to evaluate the feasibility of thermal damage assessment of blood vessels by using laser-induced release of liposome-encapsulated dye in the particular case of 805 nm laser irradiation and ICG potentiation. This new technique will be described and some results obtained in vivo in a hamster skin flap model will be presented using a specific ICG formulation (water and oil emulsion).

MATERIALS AND METHODS

Hamster Skin-Flap Window Model

This window model is a chronic preparation of the skin and provides access from the exterior and interior surfaces. All the layers are intact, from the stratum corneum, composed of keratinocytes, stratum granulosum between the stratum corneum and the basal cells, and the fascial layers of the dermis. Arterioles, with diameters up to 150 μm and venules with diameters up to 250 μm course on the fascial layers. The arterioles have circular sections while the venules can have circular or ellipsoid. The larger venules are usually ellipsoid with the major diameter parallel to the windows. The overall thickness of the preparation is approximatively 1 mm (epidermis: 30 μm; dermis 200 μm; hypodermis: 550 μm; muscular layer: 200 μm). In the epidermis there are numerous hair follicles (the hair has been shaved and remaining roots have been removed with hair depilatory cream). The color of the preparation is light yellow pink with no pigmentation in most animals. In the lower back, there are two dark pigmented flank glands (males only), but these are always kept outside the window.

The window consists of two identical and symmetric aluminium plates. This model is based on one developed previously by Papenfuss et al. [16] and slightly modified by Gourgouliatos et al. [17]. Each plate has a 18 mm central viewing aperture and are attached together surgically to provide support for the flap. The weight of each plate is 1.7 g. The implantation procedure is conducted under deep anesthesia and sterile conditions. Suture holes are used to secure the skin flap to the plates. The main steps are: (i) shaving the hair off the back of the hamster; (ii) lifting the dorsal skin flap and securing it with sutures at the top of the plate; (iii) removing one complete layer of skin and the underlying fascial and avascular tissue on the opposing side of the flap, corresponding to an area equivalent to the window aperture; and (iv) placement of the second plate over the prepared skin, and connecting and securing it to the first plate with additional sutures. Male hamsters are used since they have less fat under the skin, making the implantation procedure easier and giving a better preparation for viewing.

This model is chosen for these experiments since it is possible to distinguish two different targets: skin and blood vessels. When ICG is used, the conversion of light into heat takes place in the blood vessels. Arterioles or venules are the main targets, their size is important in regard to the laser spot and consequently the amount of ICG which can absorb the light is much more important (Fig. 1a). Concerning the skin itself, capillaries are effectively present. But, their size and their numbers are too limited to give an effective conversion of light into heat since the amount of ICG is limited by the volume of these small capillaries (Fig. 1b).

Preparation of the Dye

5,6-Carboxyfluorescein (Eastman Kodak) is used. The main excitation peak of this dye is 490 nm. The fluorescence emission is maximum at 515 nm. This carboxyfluorescein is purified using a procedure described by Ralston et al. [18]. This purification is achieved by treating with activated charcoal, washing, and finally performing hydrophobic chromatography on an Sephadex LH-20 (Pharmacia-Biotech, Uppsala, Sweden) column. Fractions are analysed by HPLC (high pressure liquid chromatography) and purified fractions are pooled together and dessicated. A 100 mM solution is prepared by dissolving a suitable amount of the purified dye in water.

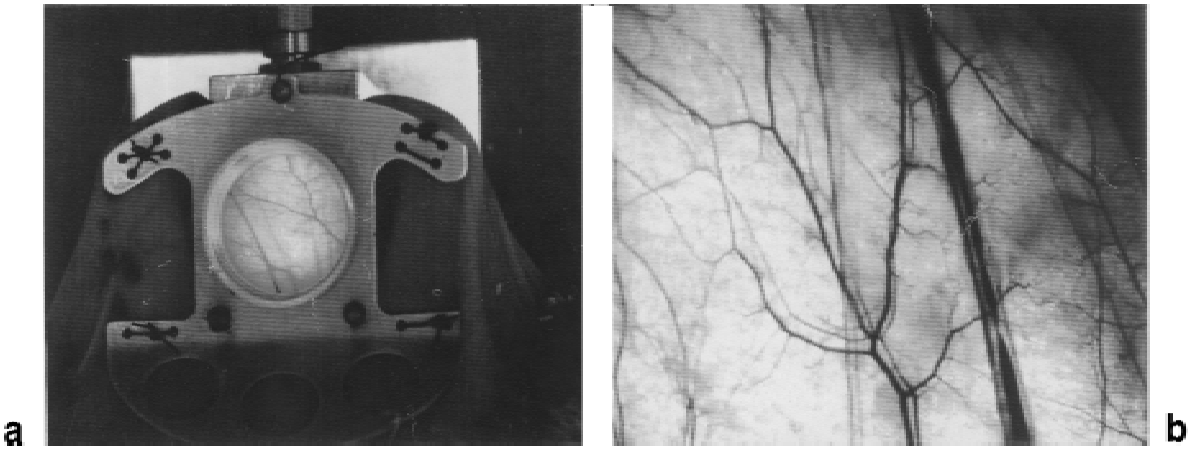


Fig. 1. **a:** Photograph of the window chamber implanted onto a hamster. Arterioles and venules can be clearly seen. **b:** Photograph of a close view of the skin flap window. Small capillaries can be distinguished inside the skin.

Preparation of Thermosensitive Liposomes

Temperature-sensitive liposomes used in this experiment are multilamellar vesicles. They are prepared by sonication, a procedure which has been described in previous articles [5,6]. A suitable amount of L-alpha-distearoyl phosphatidylcholine (DSPC: 18 carbon chains: liquid-crystal line phase transition temperature = 54°C, Lipoïd K.G., Ludwigshafen, Germany) is dissolved in chloroform (Merck, Darmstadt, Germany). The phospholipid organic solution is evaporated under reduced pressure in a rotary evaporation flask. After complete removal of chloroform, a 100 mM 5,6-CF solution is added and hydratation of lipids is carried out at 55°C for 1 hour. After an equilibration period, the lipid suspension is sonicated at 55°C during 20 min under nitrogen (Sonicator Heat-System, Sonics & Materials, Dandbury, CT) using the following parameters: 500 W, 10% output, 20,000 Hz, 3 mm diameter probe. The liposome suspension is centrifuged at 4,000 rpm for 20 min (Heraus, Hanau, Germany) in order to eliminate titanium particles. Liposomes size is determined by quasi-elastic light scattering at a 90° angle (Sematech, SM 633/RTG, Nice, France). Mean size is determined to be 300nm. The supernatant is dialyzed over 5 mM phosphate buffer saline (pH: 7.4) for 24 hours (1,000 ml, changed twice) to remove unencapsulated 5,6-CF. Immediately after dialysis, liposomes are injected to animals to prevent any leakage of the dye. Previous in vivo experiments have showed that the amount of 5,6-CF release increases in the 41-65°C range. Approximatively 50% of the fluorescence intensity is obtained at 54±2°C. For temperatures below 41 ± 2°C, no release is observed. For temperatures above 65±2°C, the maximum release is reached and consequently the maximum fluorescence intensity is reached [5,6].

Indocyanine Green Dye (ICG)

ICG (Infracyanine®, Serb, Paris-France) is used. The absorption peak of ICG in plasma or blood is approximatively 805 nm. ICG is reconstituted with sterile water immediately prior to use. Since aqueous solution of ICG in vivo is characterized by a very rapid clearance from the blood with a half-time of 3–4 min [8,19], a formulation of ICG in water and oil emulsion is used. The formulation of ICG in water and oil emulsion is performed, since small particles using phospholipids and soybean oil have been used as a long circulating emulsion system and it has been demonstrated that, in this case, the dye follows, in part, the pharmacokinetic of the particle and not only its own. The half-time of ICG o/w emulsion reaches 45 min [14].

The emulsion is prepared with phosphatidylcholine (Egg, 100% purity, Lipoïd K.G.) and soybean oil (Sigma Chemical, St. Louis, MO) by extrusion through polycarbonates filters. The content of the emulsion formulation is adjusted to obtain 5% soybean oil, 1.75% glycerol w/v final volume, and 0.6% w/v phospholipid final concentration. After addition of the chloroformic phospholipid solution to the soybean oil and mixing, this solution is evaporated under N2 stream at room temperature. Addition of glycerol and water

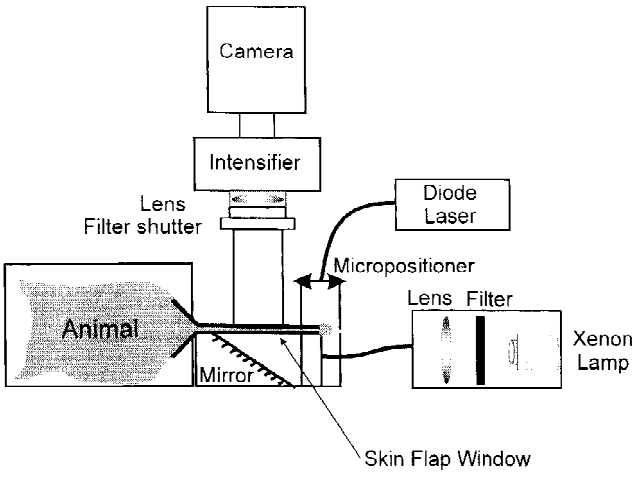


Fig. 2. A plan view of the experimental arrangement used to irradiate the window chamber and observe the fluorescence.

is performed just before ICG addition. Emulsification is carried out by extrusion through polycarbonates filters (Nuclepore, 0.2 μm diameter) 20 times.

Spectral analyses have shown that ICG interact with the interfacial layer of the emulsion leading to a shift of the maximum absorption wavelength similar to that observed in blood sample [14]. Under aseptic conditions, the hamsters are injected through the tongue vein with a bolus of the ICG emulsion at a dose of 15mg/kg usually 2 min after liposomes injection.

Fluorescence Imaging System

Fluorescence measurements are performed with a fluorescence imaging system developed in our laboratory. This system has been already described [20] (Fig. 2). Briefly, the skin flap is back-illuminated with a fiber bundle connected to a filtered Xenon lamp (490 nm interference filter, Full Width Half Maximum: FWHM = 10 nm). This wavelength corresponds to the absorption peak of the fluorescent dye 5,6-CF and provides a good vessel/tissue contrast.

Fluorescence is analysed with an ultra-high-sensitivity intensified camera (model C2400-25, Hamamatsu Photonics, Hamamatsu City, Japan) composed of a low-noise two-stage microchannel plate intensifier connected to a saticon tube. This camera is connected to an image processor (model Argus 50, Hamamatsu Photonics). Fluorescence images (512 × 512) are obtained every 40 ms on an image field of 20 mm × 20 mm (400 mm^2) at a distance of 35 cm from the skin flap. Since 5,6-CF is used, the peak fluorescence emission is selected using a narrow band interference filter (520nn, FWHM = 10 nm, Omega Optical, Brattleboro, VT). The amplification of fluorescence is calibrated to avoid any image intensifier saturation in case of intense fluorescence. Fluorescence intensities are displayed numerically (arbitrary units) by profiles or by 3-D presentation.

Diode Laser

A 805 nm diode laser, model 2372-P3 (SDL, San Jose, CA) is used. The diode laser system is used in CW (continous mode), non-contact mode (spot diameter = 1.3 mm), with a wavelength of 805 nm, a 0.8 W power, and an incident power density of 60W/cm^2. The laser energy is delivered to the target tissue by an optic fiber with a core diameter of 300 μm and a numerical aperture of 0.37. During the experiments, the distance between the tissue and the fiber is kept constant (3 mm) with a mechanical holder to give a 1.3 mm diameter spot. The laser beam profile and the beam diameter are controlled with a Beamscan (Photon Inc., Los Gatos, CA). These parameters are used in order to compare the effects of laser irradiation with emulsion and ICG.

Methods

This study consists of analyzing the fluorescence spots obtained when using an ICG formulation with o/w emulsion and different diode-laser parameters. For each hamster, the irradiance is 60W/cm^2. Pulse exposure times are increased from 1 to 6 s in order to obtain a temperature increase from the basal temperature up to 65°C, which corresponds to the maximum release of dye from the liposomes (see Fig. 3). Fluences range between 16 and 360 J/cm^2 (power = 0.8 W, spot diameter = 1.3 mm for pulse exposure time lasting from 1 s to 6 s). Eight to 10 laser pulses are performed on each skin-flap window (blood vessel or skin) 1 min to 5 min after ICG emulsion injection.

This fluorescence study is performed at a macroscopic scale. The magnification used to image the 18 mm window does not distinguish the capillaries inside the skin. One pixel represents 40 μm (20 mm/512 pixels). Only blood vessels larger than 120 μm can be clearly seen. Fluorescence intensity is evaluated with a fixed area (0.015 mm^2) corresponding to nine pixels.

Fluorescence measurements are performed from 3 min after liposomes injection (1 min after ICG injection) up to 45 min. The fluorescence is measured immediately and after each laser irradiation. The stability (particularly the rema-

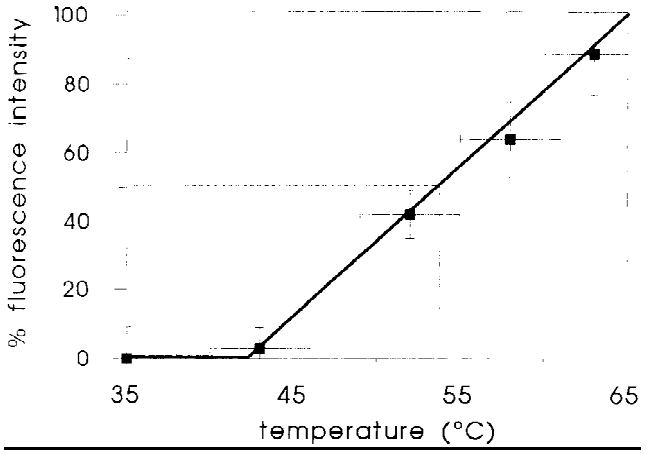


Fig. 3. In vivo temperature-dependent release of 5,6-CF from DSPC liposomes loaded with 5,6-CF. Percent of fluorescence intensity as a function of temperature (from ref. 5).

nence) of the fluorescence intensity of each spot is examined. The fluorescence measured in non-irradiated vessel is considered as the reference (0%) for calculation of relative fluorescence increase during and after laser irradiation.

RESULTS

At the beginning of the experiment (3 min after liposomes injection) an homogenous fluorescence of the skin is observed in contrast with the blood vessels which exhibits a very low fluorescence. The fluorescence intensity of non-irradiated blood vessels appears to be highly reproducible and confirms it can be considered as the background fluorescence and reference (0%) for calculation of relative fluorescence increase during and after laser irradiation. Forty-five spots are analysed on six hamsters. Data are plotted after background fluorescence substraction. At the beginning of the experiment, the homogeneous fluorescence of skin represents approximately 5% of the maximum fluorescence and decreases slowly to reach 2 and 3% after 45 min.

For fluences below 60 ± 10 J/cm^2, no fluorescence spot can be detected following laser irradiation. For fluences above 60 ± 10J/cm^2, fluorescence is observed in vessels. For fluences above 120 ± 20J/cm^2, fluorescence spots are observed on skin. Their shape and their intensities depend on both fluences and targets (blood vessel or skin). The fluorescence intensity of each spot tends to increase as a function of fluence to reach a maximum (100%) for which a truncated gaussian fluorescence profile is obtained. Figure 4 summarizes the data obtained using different fluences on both vessel and skin. The fluorescence intensities are measured immediately after the laser irradiation at the center of the spot.

For laser irradiation of blood vessel with fluences between 60 J/cm^2 and 100 ± 20 J/cm^2, an intense fluorescence at the impact and at distance of the laser impact inside the blood vessel is usually observed. It is the only case, where the fluorescence image does not give a circular fluorescence spot. Figure 5a shows an example of this observation. Figure 5b obtained 5 min later shows that the fluorescence at the distance of the laser impact has disappeared and the fluorescence of the blood vessel at this position is equivalent to that obtained before irradiation.

Different fluorescence spots can be observed as a function of fluence and tissue (blood vessel or skin). They can be classified into three categories: 1—transient fluorescence spots; 2—permanent intravascular fluorescence with transient fluo-

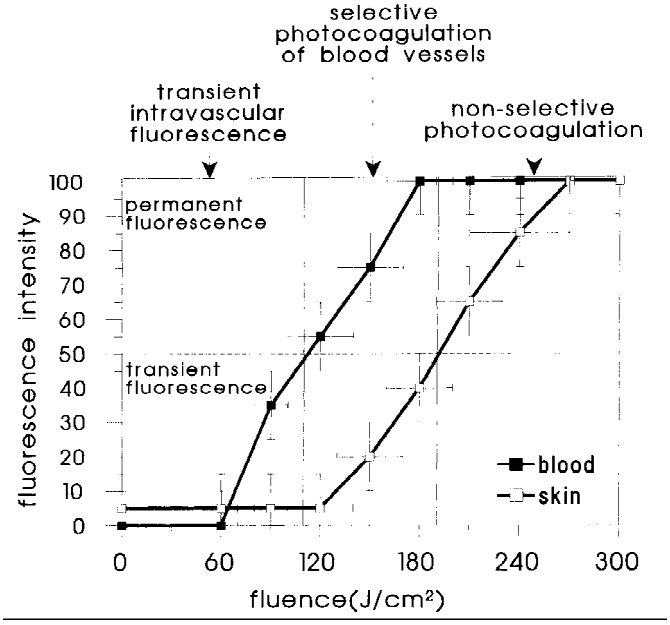
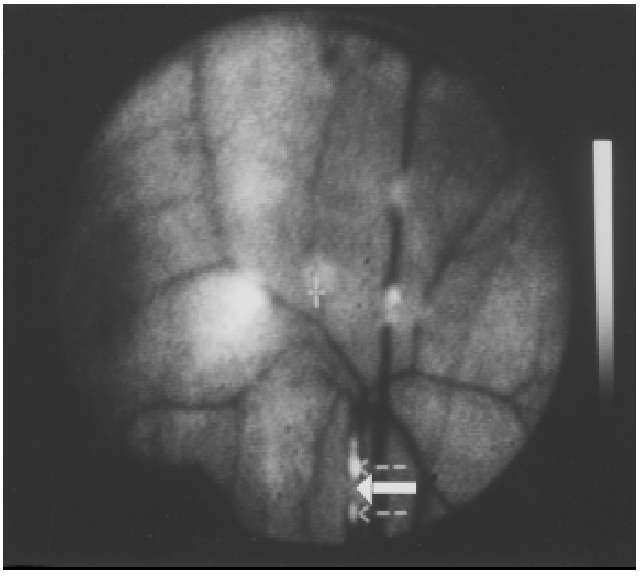


Fig. 4. Fluorescence intensity (%) as a function of fluence obtained for blood vessels larger than 200 μm and for skin. The irradiation was performed with a 0.8 μm diode laser (P=0.8W, spot diameter = 1.3 mm), incident power density of 60W/cm^2, pulse durations from 1 to 5 s). Fluorescence data were recorded 1 min after irradiation. For fluence below 60J/cm^2, only background fluorescence is observed. For fluences between 60J/cm^2 and 110J/cm^2, transient intravascular fluorescence is obtained. For fluences between 110J/cm^2 and 190J/cm^2, permanent intravascular fluorescence and transient skin fluorescence are observed. Finally, for fluences superior to 190 J/cm^2, permanent fluorescence spots are obtained for both blood vessels and skin.



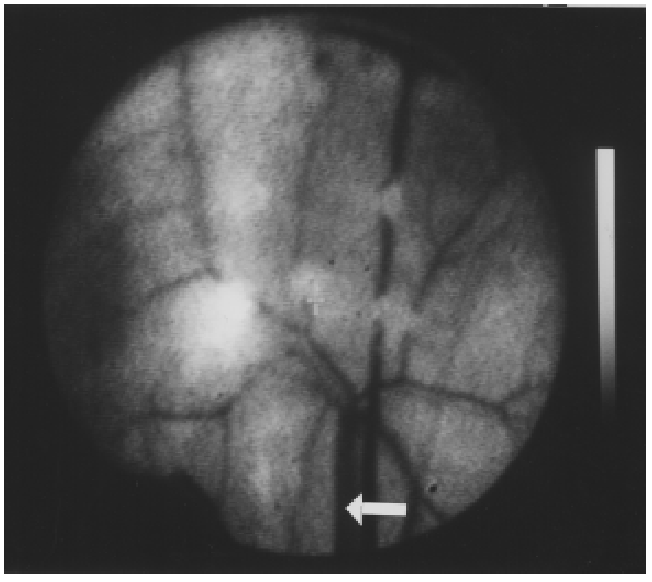
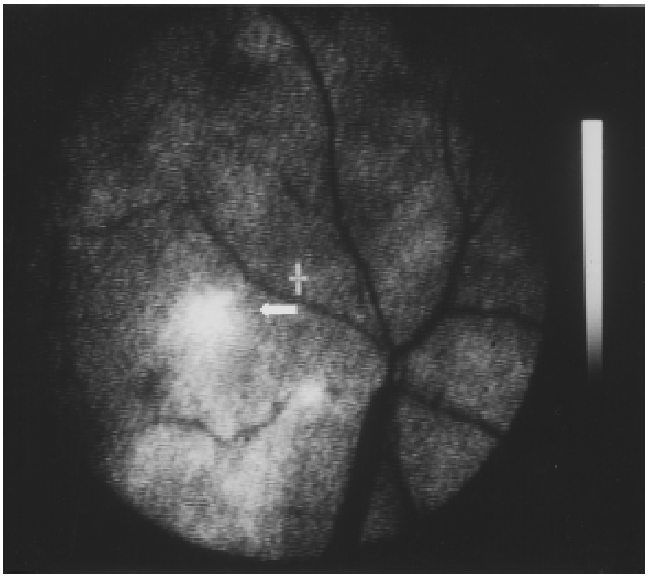


Fig. 5. **a:** Photograph of the window chamber fluorescence obtained immediately after laser irradiation of a blood vessel with 90J/cm^2. The white arrow shows that a bolus of dye is released. **b:** Photograph of the same window chamber fluorescence obtained 5 min later. The fluorescence has disappeared and the fluorescence of the blood vessel at this position is equivalent to that obtained before irradiation.



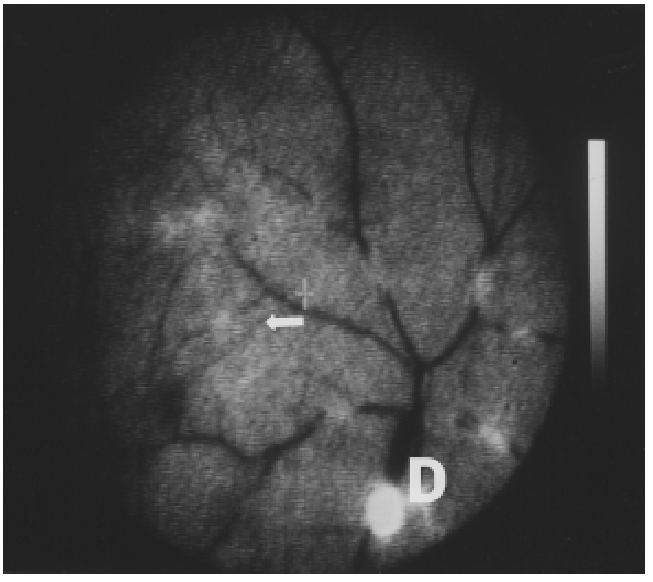


Fig. 6. **a:** Photograph of the window chamber fluorescence obtained 1 min after irradiation of skin with a fluence of 150J/cm^2. **b:** Photograph of the same window chamber. Fluorescence is recorded 30 min after the irradiation of the skin. One can note that the fluorescence spot has almost disappeared (arrow). This photograph also shows a permanent intravascular fluorescence (D) obtained with 210 J/cm^2. In that case, fluorescence is observed around the blood vessel since a temperature increase due to heat diffusion leads to a dye release in the skin.

rescence circular spot; 3—permanent circular high intensity fluorescence spot.

1. Transient fluorescence spots: these transient spots are observed on skin when using a low fluence (110 ± 20 J/cm^2 to 180 ± 20 J/cm^2). The fluorescence decreases as a function of time. Fifty percent of the initial fluorescence intensity is obtained after 40 ± 10 min (Fig. 6a,b).

2. Permanent intravascular fluorescence with transient fluorescence circular spot: these spots are observed with similar fluences but in this case, the blood vessel is the target. The fluorescence around the vessel disappears with a delay similar to that observed in the previous case (Fig. 7).

3. Permanent circular high intensity fluo-

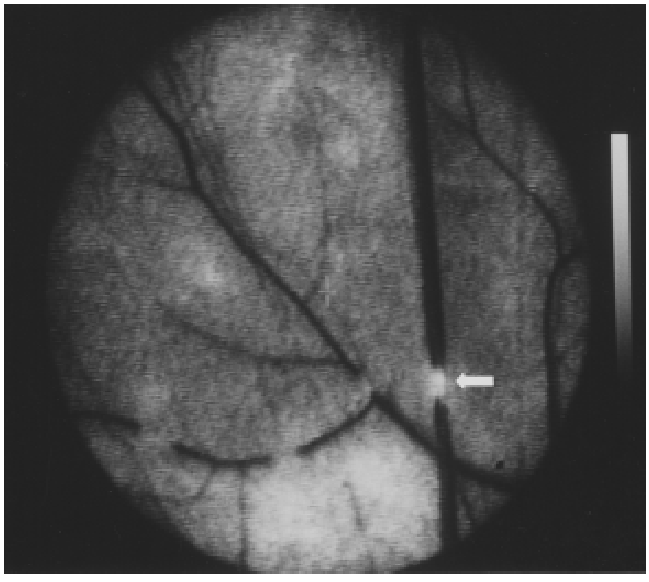


Fig. 7. Photograph of the window chamber fluorescence obtained 5 min after irradiation of a blood vessel with 150J/cm^2. The fluorescence is confined into the blood vessel (white arrow).

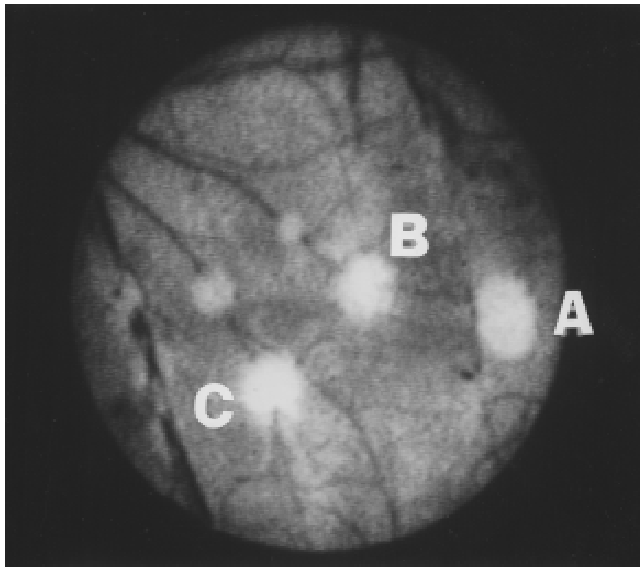


Fig. 8. Photograph of the window chamber fluorescence with three permanent fluorescence spots on the skin (A = 210J/cm^2, B = 240J/cm^2, C = 300J/cm^2).

rescence spot: these spots are systematically observed for fluence superior to 200 ± 20 J/cm^2. The maximum fluorescence intensity at the center of the fluorescence spot (gaussian distribution) reaches a maximum for a mean fluence of 270 ± 30 J/cm^2. For fluence superior to this value, the fluorescence remains stable but a truncated gaussian fluorescence profile is obtained. Figure 6b shows a fluorescence spot obtained after irradiation of a blood vessel with 210J/cm^2. Figure 8 shows a photograph of the window chamber fluorescence with three permanent fluorescence spots on the skin. (A = 210J/cm^2, B = 240J/cm^2, C = 300J/cm^2).

DISCUSSION

The mechanism of this liposome-dye system is based on the properties of temperature-sensitive liposomes. These liposomes are capable of existing in two distinct phases, the "gel" or "solid" phase and the "liquid crystalline" or "fluid" phase. The transition from the solid phase to the fluid phase is defined by a transition temperature. The transition leads to the leakage of the dye entrapped in the liposomes. The phase transition temperature corresponds to a permeability maximum due to the chain-melting phase transition. Using DSPC liposomes, we have already demonstrated that the fluorescence intensity increases linearly between 42 ± 3°C and 65 ± 3°C. Approximately 50% of the fluorescence intensity is obtained at 54°C which corresponds to the phase transition temperature of DSPC liposomes. These liposomes are confined in blood vessels. Consequently, it was proved that the release is due only to the tissue temperature increase, except for a small release due to an interaction with proteins and lipoproteins giving a background fluorescence which occurs immediately after liposomes injection. After this fast release, the dye is rapidly cleared from the circulation. If fluorescein remains essentially intravascular during the passage through the retinal circulation, in skin capillaries, however, it leaks rapidly into the surrounding extravascular compartment and this leakage is remarkably uniform [21]. Consequently, the blood vessels appear as negative contrast within the area of highest light intensity since the fluorescein half-life in blood is approximately 5 min and the fluorescein half-life of skin reaches 40 min [22].

In order to study the tissue thermal damage due to an increase of tissue temperature, the absorption of the laser light by the dye loaded in the liposome has to be avoided, making the choice of a fluorescent dye and a wavelength crucial. The absorption of 5,6-CF is maximum at 490 nm, but there is no absorption in the red and infrared. Using a diode laser emitting at 805 nm, this constraint is respected.

Our results show that the dye is released in response to laser energy and consequently to a temperature increase. The release occurs despite the fact that the liposomes do not have an absorbing dye entrapped within their aqueous compartment. With a 0.8 W power, and a previous 15 mg/kg ICG injection, the fluorescence emission shows a linear dependence on fluence between 60 and 180 J/cm^2 for blood vessel and 120 and 270 J/cm^2 for skin. The special property of ICG is that its wavelength of peak absorption matches nearly identically to the peak emission of the diode laser. In case of ICG in aqueous solution, ICG-enhanced laser photocoagulation studies have shown that potentiation of the diode laser's effect depends on the delay between i) administration of ICG and laser irradiation, and ii) target tissue. Using o/w emulsion, our study does not demonstrate any influence of the ICG half-life on the conversion of the laser light in a time window of 1 to 5 min after injection. Since the ICG half-life of this solution is 40 min [14], the influence (theoretically 10% decrease of the ICG absorption) must exist. The target (blood vessel or skin) and particularly the size of the blood vessel also play a role on the efficacy of the light conversion. This can be easily understood since the conversion of light into heat depends on the amount of ICG in the target.

The observation of an intense fluorescence at a distance of the laser impact when blood vessels are irradiated with fluences between 60 J/cm^2 and 100 J/cm^2 is explained by the release of a bolus of dye by the liposomes. In that case, due to the low fluence, the temperature inside the vessel is above 42°C but remains below the critical coagulation temperature. Since the endothelium is undamaged, no extravasion of the dye is observed. In fact, this observation corresponds to the laser-triggered release concept already described by Khoobehi and Peyman for the measurement of retinal blood flow [23]. They used highly concentrated fluorescent dye encapsulated into temperature-sensitive liposomes which were injected intravenously. When these liposomes were exposed to the heat pulse generated by a laser, hemoglobin and the highly concentrated dye inside the liposome absorbed the heat pulse. Liposomes leaked and the dye was diluted and fluoresced. Then, they traced the released dye front to measure the blood flow velocity. Usually 30 sec later, the dye is washed out and no fluorescence can be detected.

For fluences above 110J/cm^2, different fluroescence spots can be observed as a function of fluence and tissue (blood vessel or skin). The transient spot observed on skin when using a low fluence (Fig. 6a,b) can be explained by an extravasion of dye from capillaries since their endothelium is damaged very quickly [24]. However, in this case, the temperature of the skin remains below the critical coagulation temperature. The critical coagulation for skin in the human is considered to be 55°C [25]. Since the skin is undamaged, the perfusion process is intact enabling the dye to be cleared from the tissue. The decrease of fluorescence depends on the fluorescein half-life in skin which reaches 40 min [22].

With similar fluences, permanent intravascular fluorescence is observed in blood vessels. First, the endothelium is damaged giving an extravasation of dye around the vessel. With increasing fluence from 100 J/cm^2 up to 190J/cm^2, the critical coagulation temperature is reached inside the blood vessel (Fig. 7). Then the dye is definitively trapped inside the blood vessel and cannot be cleared. Around the blood vessel, transient fluorescence circular spots are observed for a delay similar to that observed in the previous case (Fig. 6b).

At last, for fluence superior to 190 ± 20 J/cm^2, permanent fluorescence circular spots are observed. In a similar manner, at the beginning of the irradiation, the vessel or the capillary wall is damaged first and consequently the dye leaks outside. With increasing fluence, temperature becomes superior to the critical coagulation temperature for both blood vessel and skin. The dye cannot escape from the modified protein environment and the fluorescence stays during the whole experiment. In that case non-selective coagulation is observed. Maximum fluorescence intensity at the center of the fluorescence spot (gaussian distribution) is obtained for a mean fluence of 270 ± 20 J/cm^2 for the skin. This corresponds to the maximum of dye release from liposomes and consequently a tissue temperature of 65±3°C. For higher fluences, the maximum fluorescence remains stable but a truncated gaussian fluorescence profile is obtained. For these fluences, one can consider that dye release from the liposomes is maximum (Fig. 8, fluorescence spot C). An increase of temperature causes, in fact, further lysing of surrounding liposomes because of the transfer of heat to the surrounding area. This was observed in our previous studies and by Khoobehi et al. [26].

To summarize, the advantages of this liposome-dye system are 1) direct measurements can

be obtained; (2) several repeated readings can be made from one injection; 3) continuous monitoring of the fluorescence can be made; 4) temperature-sensitive range can be adopted using different liposomes compositions; 5) circulation times of several hours can be achieved using DSPC liposomes, 6) the tissue microcirculation and the vessel macrocirculation can be investigated simultaneously, therefore changes in response to a treatment regimen and/or ICG formulations can be detected. One main constraint exists: the fluorescent dye encapsulated into the liposomes has to be carefully chosen in order to avoid any direct absorption by the dye itself.

In conclusion, one of the most significant applications of this experimental technique is the evaluation of various degrees of tissue thermal damage. It could be possible to consider the application of this technique in Ophthalmology and Dermatology, and possibly for the evaluation of burn injury.

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